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Preparation of flavocoenzyme isotopologues by biotransformation of purines

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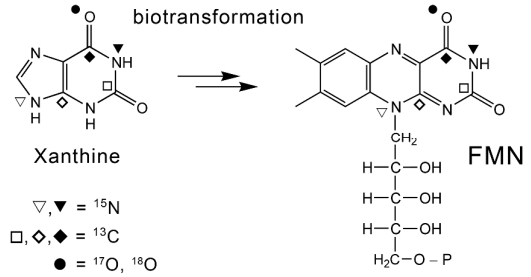


Abstract

Isotope-labeled flavins are crucial reporters for many biophysical studies of flavoproteins. A purine-deficient *Escherichia coli* strain engineered for expression of the *ribAGH* genes of *Bacillus subtilis* converts isotope-labeled purine supplements into the riboflavin precursor, 6,7-dimethyl-8-ribityllumazine, with yields up to 40 %. The fermentation products can subsequently be converted into isotope-labeled riboflavin and the cognate flavocoenzymes, FMN and FAD, by *in vitro* biotransformation with better than 90 % yield. Using this approach, more than 100 single or multiple ^{13}C, ^{15}N, ^{17}O and ^{18}O-labeled isotopologues of these cofactors and ligands become easily accessible enabling advanced ligand-based spectroscopy of flavoproteins and lumazine receptor proteins at unprecedented resolution.

Introduction

Flavocoenzymes are absolutely required in all organisms where they serve as cofactors for a wide variety of redox reactions involving one-electron and two-electron reactions.[1] Moreover, various non-redox proteins including certain dehydrogenases, DNA repair enzymes, optical transponders and blue light receptors depend on flavin cofactors.[2-4]

Various spectroscopic techniques used for the investigation of flavoproteins, such as NMR, EPR and IR spectroscopy benefit from the introduction of stable isotope labels into the flavin cofactors. Specifically, ^{13}C and ^{15}N labelings result in substantial sensitivity gains for ^{13}C and ^{15}N NMR, and may be exploited for signal enhancement in photochemically-induced dynamic nuclear polarization NMR (photo-CIDNP NMR).[5-7] To probe electronic structures of organic molecules via the detection of hyperfine couplings using EPR-derived hyperfine spectroscopies such as ENDOR[8,9], ESEEM[10], or HYSCORE[11], (i) ^{12}C-to-^{13}C replacement is indispensable because of these two isotopes only ^{13}C carries a nuclear spin, and (ii) ^{14}N-to-^{15}N substitution provides spectral simplification due to the lack of a quadrupole moment of ^{15}N, and means to unambiguously assign nitrogen hyperfine couplings due to the different strength

of the interaction of the unpaired electron spin with ^{14}N and ^{15}N. Both labelings are furthermore prerequisite for vibrational assignments in IR spectroscopy.

Methods for chemical and for enzyme-assisted synthesis of flavocoenzymes with site-specific stable-isotope labeling have been developed over a period of several decades (for review see[12]). Notably, certain isotopologues carrying ^{13}C and/or ^{15}N in the pyrimidine and/or pyrazine rings of the isoalloxazine chromophore can be obtained with relative ease by chemical synthesis using isotopologues of barbituric acid as synthones.[13,14] The benzenoid ring of flavins can be labeled with ^{13}C by enzyme-assisted synthesis using commercially available isotopologues of glucose as synthones.[15] More recently, a recombinant *Escherichia coli* strain that had been engineered to express the *rib*A, *rib*G and *rib*H genes of *Bacillus subtilis* was used to convert universally or specifically ^{13}C-labeled glucose into 6,7-dimethyl-8-ribityllumazine, the direct biosynthetic precursor of riboflavin.[16] Depending on the label distribution in the proffered glucose, fermentation afforded the lumazine derivative either with universal ^{13}C labeling or as a mixture of different isotopologues that can be transformed into riboflavin with near-quantitative yield by enzyme-mediated biotransformation *in vitro* (cf. [17]for details). However, riboflavin isotopologues carrying single ^{13}C labels in either the 4 or 10a position and isotopologues carrying single ^{15}N labels in either the 1 or 3 position of the isoalloxazine chromophore are not accessible by current technology but could be particularly useful for certain spectroscopic experiments. In this paper, we describe the *in vivo* biotransformation of isotope-labeled purines into isotope-labeled 6,7-dimethyl-8-ribityllumazine (**6**, cf. Scheme 1) resp. riboflavin. Since selective labeling is technically more easily achieved for purines than for isoalloxazines, this opens an elegant and affordable approach to the synthesis of numerous single- or multiple-labeled flavin isotopologues that can now be exploited as spectroscopic probes for investigation of a large group of structurally and functionally diverse flavoproteins.

For practical reasons, the *in vivo* biotransformation of purines in this study was designed to yield the riboflavin precursor, 6,7-dimethyl-8-ribityllumazine (**6**, cf. Fig. 1) (rather than riboflavin) as fermentation product, which is subsequently converted into riboflavin, FMN or FAD by enzyme-mediated *in vitro* biotransformation. Notably, however, **6** is not only an intermediate of riboflavin biosynthesis but has also been found to serve as protein cofactor in its own right. Specifically, **6** is used as chromophore by lumazine protein, which acts as optical transponders in bacterial luminescence and modulates both the frequency range and the quantum yield of emitted light (for review see [18]). More recently, **6** has been shown to serve as antenna chromophore in certain DNA photolyases and as a ligand for receptors used by the subclass of human mucosa-associated T-cells (MAIT cells).[19,20] Hence, isotopologues of **6** which are generated in the present work have the potential to contribute to the investigation of these more recently discovered receptor proteins. Notably, the present method can afford isotopologues of **6** which are not accessible by prior technology. It also provides convenient access to many multiply labelled isotopologues of **6** as well as riboflavin, FMN and FAD.

Results and Discussion

The biosynthesis of riboflavin starts from GTP (for review see [21]). By a sequence of six enzyme-catalyzed reactions, the carbon and nitrogen atoms of GTP except C-8 and the carbonyl oxygen in the 4 position (designated a – i in Fig. 1) are incorporated into the vitamin, where they constitute the pyrimidine ring, the pentityl side chain and parts of the pyrazine ring, whereas the carbon atoms of the xylene moiety are derived from the pentose phosphate pool via 3,4-dihydroxy-2-butanone 4-phosphate (**8**, Scheme 1). In order to recruit that pathway for the preparative conversion of isotope-labeled purines into the riboflavin precursor **6**, a purine-deficient *E. coli* mutant (carrying a *pur*F deletion) was endowed with a plasmid directing the expression of the *ribAGH* genes of the *Bacillus subtilis rib* operon under the

control of a T5 promoter and *lac* operator (the *B. subtilis rib* operon used comprises the *rib*ABGH genes, but the *ribB* gene specifying riboflavin synthase has been inactivated by an F2A mutation conducive to the production of a full length protein devoid of catalytic activity).[22] Whereas the recombinant *rib* operon does not supply catalytically active riboflavin synthase, a limited amount of active riboflavin synthase is provided by transcription/translation of the chromosomal *ribC* gene of the *E. coli* host under the control of its natural promoter (somewhat unfortunately, the gene specifying riboflavin synthase is designated *rib*B in *B. subtilis* but *rib*C in *E. coli*). When grown with purine supplements (hypoxanthine (**1**), xanthine (**3**) or adenine), **6** is accumulated in the culture medium reaching concentrations up to 0.3 mM (Fig. 2); riboflavin is also accumulated, albeit in lower concentration, up to about 50 μM.

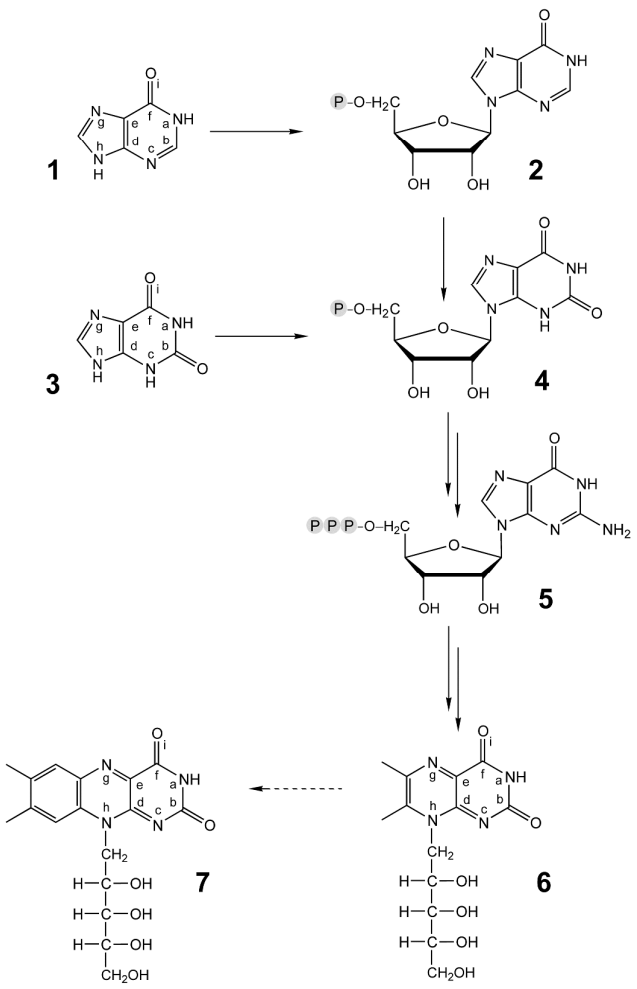


Fig. 1. Bioconversion of purine bases by the recombinant *E. coli* strain PL919. Due to deletion of the *pur*F gene, the strain is unable to produce purines *de novo*. Biosynthetically equivalent atoms are marked with letters a – i.

Since the recombinant *E. coli* strain has an absolute requirement for a purine source due to deletion of the *pur*F gene, isotope-labeled purines are biosynthetically diverted to **6** and to riboflavin without dilution. Due to the enhanced expression of riboflavin biosynthesis enzymes, labeled purines are efficiently converted into **6**, with yields up to 40 % based on purine. Concentrations up to 0.3 mM **6** (i.e. about 100 mg of **6** per liter of culture medium) can be obtained in shaking cultures with glucose as carbon source (Fig. 2). However, it should be noted that the concentration of **6** declines rapidly after reaching its maximum at early

stationary phase; timely termination of the fermentation is therefore essential. Besides **6**, the fermentation broth also contains a minor amount of isotope-labeled riboflavin (generated by the activity of the chromosomally encoded riboflavin synthase). Riboflavin and **6** can be harvested easily by adsorption to Florisil® which retains both compounds with remarkable selectivity. Subsequent to separation by cation exchange chromatography, riboflavin and **6** can be crystallized from water. Using various ^{13}C-, ^{15}N-, ^{17}O- or ^{18}O-labelled xanthine or hypoxanthine substrates (Table 1), the resulting **6** and riboflavin isotopologues were characterized by NMR and LC-MS techniques as shown in the Supporting Information (Tables S1-S6).

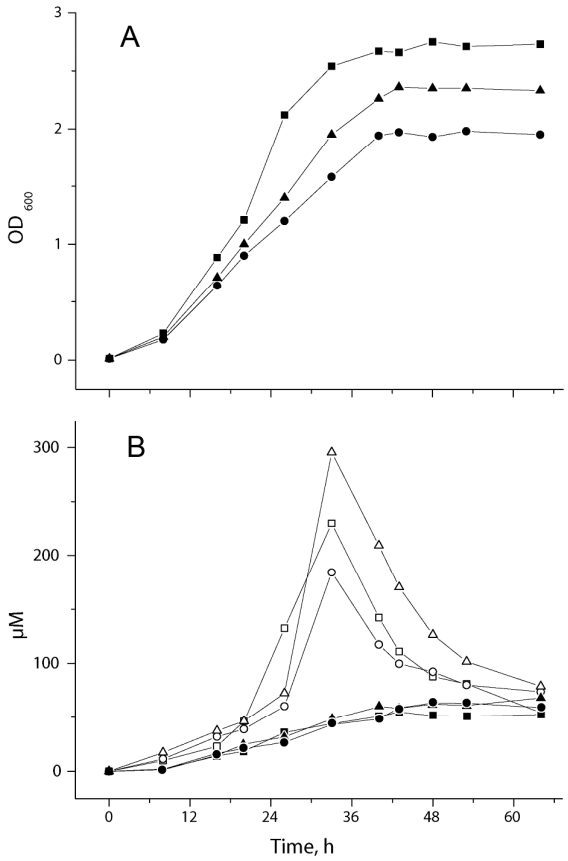
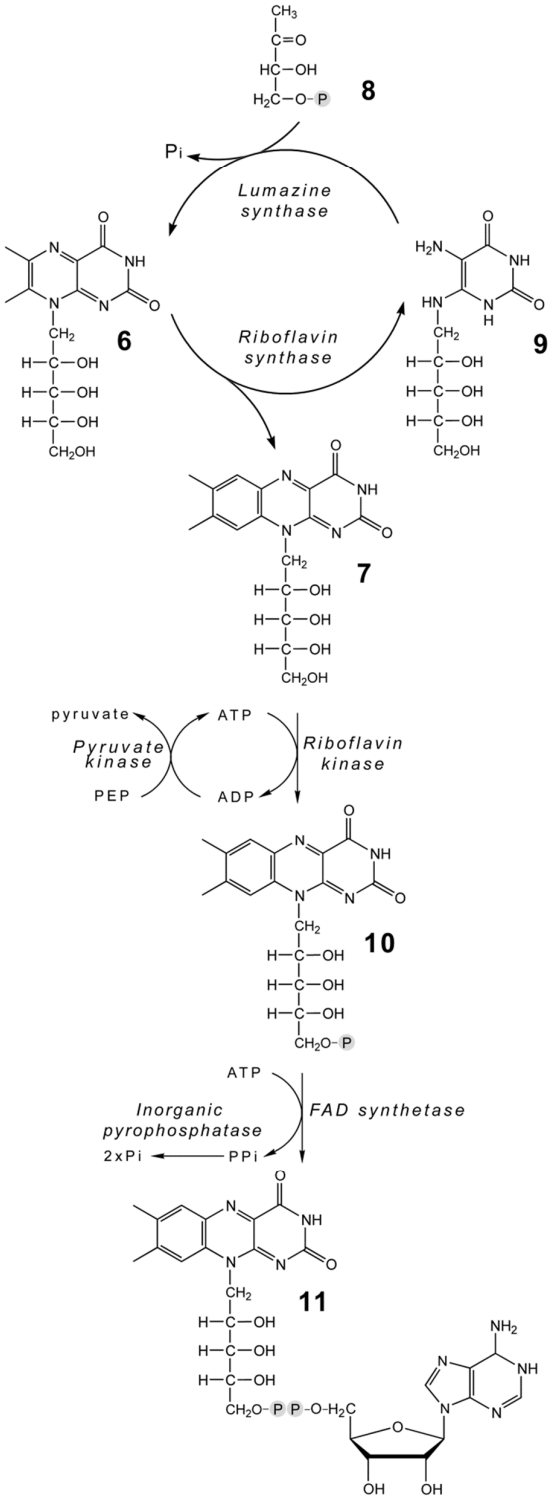


Fig. 2. (**A**) Growth of *E. coli* PL919 in minimal medium with supplements (80 mg/L) of adenine (■), xanthine (●) or hypoxanthine (▲); OD_{600}, optical density at 600 nm. (**B**) accumulation of 6,7-dimethyl-8-ribityllumazine (open symbols) and riboflavin (closed symbols).

The riboflavin precursor **6** can be converted into riboflavin with near-quantitative yield by enzyme catalysis under strictly anaerobic conditions (required to protect the oxygen-sensitive intermediate **9**). Briefly, as shown in Scheme 1, **6** is converted into a mixture of riboflavin and **9** by riboflavin synthase, and **6** is then regenerated from **9** by lumazine synthase using **8** (prepared from ribose 5-phosphate) as second substrate. Riboflavin can be recovered directly in crystalline form from the reaction mixture. Residual product can be recovered from the mother liquor by cation exchange chromatography. Alternatively, the crude riboflavin obtained from **6** can also be used directly as substrate for the enzyme-assisted preparation of FMN or FAD.

We also report optimized protocols for the conversion of isotope-labeled riboflavin into the cognate FMN or FAD isotopologues. Near-quantitative yields can be obtained by recycling the ATP serving as phosphate donor (Scheme 1). Moreover, the formation of FAD is facilitated by the inclusion of inorganic pyrophosphatase in the reaction mixture. Due to the use of the auxiliary enzymes, the spent reaction mixture contains only pyruvate, FMN or FAD, catalytic amounts of adenosine nucleotides, and protein, which can be removed by ultrafiltration. In many cases, the crude reaction mixtures can be used directly for the reconstitution of apo-flavoproteins in order to generate samples for physical studies, without prior purification. Alternatively, FMN resp. FAD can be purified by hydrophobic chromatography.



Scheme 1.

Methods for the synthesis of xanthine and hypoxanthine are very well documented in the literature and enable the preparation of virtually all conceivable single-labeled and

multiple-labeled isotopologues from simple isotope-labeled synthones such as cyanide, bromoacetate, ammonia and/or nitrite that are all commercially available at moderate costs.[23-34] Hence, numerous isotopologues of **6** and of riboflavin carrying single or multiple labels in the heterocyclic part of the respective chromophores can now be prepared as desired for specific spectroscopic problems (cf. Tables 1 and 2). Moreover, it is possible to predetermine the label distribution of the heterocyclic chromophore moiety by the labeling pattern of a purine precursor and to simultaneously introduce ^{13}C labeling to the carbocyclic part of the chromophore by culturing the recombinant *E. coli* strain on ^{13}C-labeled glucose in conjunction with a labeled or unlabeled purine precursor. As an example, we demonstrate the preparation of a riboflavin isotopologue with comprehensive ^{13}C labeling of the benzenoid ring but with an unlabeled pyrimidine ring by growing the recombinant *E. coli* strain with unlabeled hypoxanthine as purine source and [U-$^{13}C_6$]glucose as carbon source; the yield of [6α,6,7,7α,1',2',3',4',5'-$^{13}C_9$]-**6**, based on [U-$^{13}C_6$]glucose, is about 1.0 %. Notably, multiple-labeled "designer isotopologues" can open new approaches to the investigation of polarization transfer pathways in free radical states of flavoproteins. Using this combinatorial approach, more than 100 of single or multiple ^{13}C, ^{15}N, ^{17}O and ^{18}O-labeled isotopologues of these cofactors and ligands become easily accessible enabling advanced ligand-based spectroscopy of flavoproteins and lumazine receptor proteins at unprecedented resolution.

Table 1. Conversion of purine isotopologues into 6,7-dimethyl-8-ribityllumazine (**6**).

Purine Supplement	Carbon source	Product	Yield % [a]	Isotope enrichment [b]
[4-$^{13}C_1$]xanthine	glucose	[8a-$^{13}C_1$]-**6**	11	> 97
[6-$^{13}C_1$]xanthine	glucose	[4-$^{13}C_1$]-**6**	8	> 97
[9-$^{15}N_1$]xanthine	glucose	[8-$^{15}N_1$]-**6**	10	94
[2-$^{13}C_1$]xanthine	glucose	[2-$^{13}C_1$]-**6**	24	> 97
[1-$^{15}N_1$]xanthine	glucose	[3-$^{15}N_1$]-**6**	10	> 97
[6-$^{17}O_1$]hypoxanthine	glucose	[4-$^{17}O_1$]-**6**	32	67 [d]

[6-$^{18}O_1$]hypoxanthine	glucose	[4-$^{18}O_1$]-**6**	41	89 [e]
hypoxanthine	[U-$^{13}C_6$]glucose	[6,6α,7,7α,1',2',3',4',5'-$^{13}C_9$]-**6**	1 [c]	>97 [f]

[a] Based on proffered purine, mol/mol. [b] Isotope enrichment was determined as described in Supporting Information, pp. 40-44. [c] Based on proffered [U-$^{13}C_6$]glucose. [d] Isotope enrichment of $H_2{}^{17}O$ used as starting material for hypoxanthine production was 75 %. [e] Isotope enrichment of $H_2{}^{18}O$ as starting material for hypoxanthine production was 95 %. [f] Isotope enrichment of [U-$^{13}C_6$]glucose used for fermentation was 99 atomic %.

Experimental Section

Materials. Xanthine isotopologues were obtained by published procedures.[23-34] [6-$^{17}O_1$]Hypoxanthine and [6-$^{18}O_1$]hypoxanthine were obtained from 6-bromopurine as described in Supporting Information.

Recombinant riboflavin synthase (*E. coli*), lumazine synthase (*B. subtilis*), 3,4-dihydroxybutanone 4-phosphate synthase (*E. coli*), riboflavin kinase (*Schizosaccharomyces pombe*), and flavokinase/FAD-synthetase (*Corynebacterium ammoniagenes*) were prepared by published procedures.[17,35,36]

Bacterial strain. A purine-deficient *E. coli* strain JC182 (λ^-, e14$^-$, *pur*F1, *thi–1*) (*E. coli* Genetic Stock Center, Yale University, New Haven, CT) was transformed with plasmids pRFN4 [16] and pREP4 [37] affording the recombinant strain PL919.

[8a-$^{13}C_1$]6,7-Dimethyl-8-ribityllumazine. *E. coli* strain PL919 was grown in LB medium with ampicillin (50 mg/L) and kanamycin (15 mg/L) overnight. The cells were harvested and resuspended in 1 L of culture medium containing 12 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 2 g of NH_4Cl, 60 mg of $MgSO_4$, 30 mg of $CaCl_2$, 5 g of glucose, 5 g of Casamino Acids (Difco Laboratories GmbH, Augsburg, Germany), 30 mg of ampicillin, 80 mg of [4-$^{13}C_1$]xanthine, 4 mL of vitamin solution (containing, per ml, pyridoxamine hydrochloride, 0.08 mg, thiamine hydrochloride, 0.04 mg, *p*-aminobenzoate, 0.08 mg, calcium pantothenate, 0.08 mg, biotin, 0.02 mg, folic acid, 0.04 mg, nicotinamide, 0.06 µg, cyanocobalamine, 0.4 mg) and 1 mL of trace element solution (containing, per mL, $FeSO_4$, 4.5 mg, $MnCl_2$, 8.5 mg,

$ZnSO_4$, 0.5 mg, $CuCl_2$, 0.15 mg, $NiCl_2$, 0.1 mg, $CoCl_2$, 0.05 mg, H_3BO_3, 0.1 mg). The suspension was incubated overnight with shaking at 30 °C. The cells were pelleted, and the supernatant was passed through a column of Florisil (3.0 × 10 cm) that was subsequently washed with 0.4 L of water and developed with 50 % aqueous acetone containing 10 mM NH_4OH. The effluent was concentrated to dryness under reduced pressure. The residue was dissolved in 50 mL of water. The solution was centrifuged, and the supernatant was passed through a column of AG 50 WX8 (200 – 400 mesh, H^+ form, 1 × 20 cm) that was then developed with water. Green-fluorescent fractions were combined and concentrated to dryness under reduced pressure affording 22.4 mg (69 μmol) of 6,7-dimethyl-8-ribityllumazine (yield, 11.4 % based on proffered xanthine). Yellow-fluorescent fractions were also combined and concentrated affording 1.3 mg (3.5 μmol) of riboflavin. The products were crystallized from water. ^{13}C enrichment at position 8a carbon atom was > 97 % (LC-MS).

[4-$^{13}C_1$]6,7-Dimethyl-8-ribityllumazine. The compound was prepared from 100 mg of [6-$^{13}C_1$]xanthine as described above. Yield, 20 mg, 8.2 % based on proffered xanthine. ^{13}C enrichment at position 4 carbon atom was >97 % (LC-MS).

[2-$^{13}C_1$]6,7-Dimethyl-8-ribityllumazine. The compound was prepared from 100 mg of [2-$^{13}C_1$]xanthine as described above. Yield, 20 mg, 8.2 % based on proffered xanthine. ^{13}C enrichment at position 4 carbon atom was >97 % (LC-MS).

[8-$^{15}N_1$]6,7-Dimethyl-8-ribityllumazine. The compound was prepared from 95 mg of [9-$^{15}N_1$]xanthine as described above. Yield, 22 mg, 10 % based on proffered. ^{15}N enrichment at position 8 nitrogen atom was 94 % (LC-MS).

[3-$^{15}N_1$]6,7-Dimethyl-8-ribityllumazine. The compound was prepared from 16 mg of [1-$^{15}N_1$]xanthine as described above. Yield, 3.3 mg, 10 % based on proffered xanthine. ^{15}N enrichment at position 3 nitrogen atom was 96 % (LC-MS).

[4-$^{17}O_1$]6,7-Dimethyl-8-ribityllumazine. The compound was prepared from 43 mg of [6-$^{17}O_1$]hypoxanthine as described above. Yield, 33 mg, 32 % based on proffered hypoxanthine. ^{17}O enrichment at position 4 carbonyl group was 67 % (LC-MS).

[4-$^{18}O_1$]6,7-Dimethyl-8-ribityllumazine. The compound was prepared from 60 mg of [6-$^{18}O_1$]hypoxanthine as described above. Yield, 59 mg, 41 % based on proffered hypoxanthine (mol per mol). ^{18}O enrichment at position 4 carbonyl group was 87 % (LC-MS).

[6*α*,6,7,7*α*,1',2',3',4',5'-$^{13}C_9$]6,7-Dimethyl-8-ribityllumazine. Fermentation was performed as described above using [U-$^{13}C_6$]glucose (3 g) and unlabeled hypoxanthine (100 mg, Sigma-Aldrich, Taufkirchen, Germany) as supplement, per liter. Yield, 55 mg, 1.03 % based on proffered [U-$^{13}C_6$]glucose.

3,4-Dihydroxy-2-butanone 4-phosphate. The following procedure is based on a method described earlier.[38] A reaction mixture (40 mL) containing 100 mM Tris hydrochloride, pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 10 mM D-ribose 5-phosphate, 25 units of phosphoriboisomerase from spinach (Sigma, Taufkirchen, Germany) and 30 units of 3,4-dihydroxybutanone 4-phosphate synthase was incubated at 37 °C. The pH value of the reaction mixture was kept at 8.0 by adding aliquots of 5 M NaOH. After six h, the reaction mixture was passed through an Ultracell ultrafiltration disc NMWL 10 kDa (Merck-Millipore, Darmstadt, Germany). Ethanol (96%, 200 ml) was added to the filtrate, and the mixture was centrifuged (4500 × g, 4 °C, 30 min). Barium acetate (690 mg) was added to the supernatant. After incubation at -20 °C overnight, the sample was centrifuged (4500 × g, 4 °C, 30 min). The pellet was resuspended in 5 ml of water. Sodium sulfate (57 mg) was added, and the mixture was centrifuged (1000 × g, 4 °C, 10 min). The supernatant containing 3,4-dihydroxy-2-butanone 4-phosphate was stored in 0.5 ml aliquots at -80 °C. Universally ^{13}C-labeled 3,4-dihydroxy-2-butanone-4-phosphate was prepared as described earlier.[38]

[10a-$^{13}C_1$]Riboflavin. A reaction mixture containing 50 mM Tris hydrochloride, pH 7.0, 100 mM NaCl, 5 mM dithiothreitol, 60 mg of [8a-$^{13}C_1$]6,7-dimetyl-8-ribityllumazine, 36 mg of

3,4-dihydroxy-2-butanone-4-phosphate, 33 units of riboflavin synthase and 33 units of lumazine synthase in a total volume of 25 ml of was incubated at 37 °C. The formation of riboflavin was monitored photometrically at 470 nm (ε_{470} = 9,600 M^{-1} cm^{-1}). The yellow precipitate was harvested by centrifugation, washed with water and dried over phosphorus pentoxide. Yield, 62.4 mg, 92 % based on proffered **6**.

Other riboflavin isotopologues ([2-$^{13}C_1$]-**7**, [4-$^{13}C_1$]-**7,** 10-$^{15}N_1$]-**7,** [3-$^{15}N_1$]-**7,** [4-$^{17}O_1$]-**7,** [4-$^{18}O_1$]-**7,** [5a,6,7,7α,8,8α,9,9a,1',2',3',4',5'-$^{13}C_{13}$]-**7**) were synthesized using the same protocol. The starting amounts of the **6** and **8** as well as the yield of **7** are shown in Table 2.

Table 2. Synthesis of riboflavin isotopologues.

Substrate A	Substrate B	Product	Yield, % [a]	Isotope enrichment [b]
[8a-$^{13}C_1$]-**6** (60 mg)	**8** (36 mg)	[10a-$^{13}C_1$]-**7** (62.4 mg)	92	> 97
[2-$^{13}C_1$]-**6** (10 mg)	**8** (6.0 mg)	[2-$^{13}C_1$]-**7** (12.0 mg)	96	> 97
[4-$^{13}C_1$]-**6** (10 mg)	**8** (6.0 mg)	[4-$^{13}C_1$]-**7** (12.2 mg)	96	> 97
[8-$^{15}N_1$]-**6** (10 mg)	**8** (6.0 mg)	[10-$^{15}N_1$]-**7** (12.5 mg)	96	94
[3-$^{15}N_1$]-**6** (3.0 mg)	**8** (1.8 mg)	[3-$^{15}N_1$]-**7** (3.3 mg)	96	> 97
[4-$^{17}O_1$]-**6** (4.5 mg)	**8** (2.7 mg)	[4-$^{17}O_1$]-**7** (5.4 mg)	95	67
[4-$^{18}O_1$]-**6** (5.0 mg)	**8** (3.0 mg)	[4-$^{18}O_1$]-**7** (5.5 mg)	95	89
[6,6α,7,7α,1',2',3',4',5'-$^{13}C_{13}$]-**6** (5.0 mg)	[U-$^{13}C_4$]-**8** (3.0 mg)	[5a,6,7,7α,8,8α,9,9a,1',2',3',4',5'-$^{13}C_{13}$]-**7** (5.6 mg)	95	>97

[a] Based on proffered **6**, mol/mol. [b] Isotope enrichment was determined as described in Supporting Information, pp. 40-44.

[10a-$^{13}C_1$]-FMN from [10a-$^{13}C_1$]riboflavin. [10a-$^{13}C_1$]Riboflavin (54 mg) was suspended in 22 ml of 100 mM Tris hydrochloride, pH 8.0, containing 5 mM $MgCl_2$, 5 mM DTT, 0.02% NaN_3, 0.5 mM ATP, 8.5 mM sodium phosphoenol pyruvate, 55 units of pyruvate kinase from rabbit muscle and 20 units of riboflavin kinase. The mixture was incubated at 37 °C for 12 h. The pH value of the reaction mixture was kept at 8.0 by the addition of 5 M NaOH as

required. The progress of the reaction was monitored by thin layer chromatography (microcrystalline cellulose plates, Macherey-Nagel; eluent, 350 mM phosphate, pH 7.0). Typically, riboflavin was completely converted to FMN after 12 h. Yield, 62 mg, 94 % based on proffered riboflavin. The solution was passed through an Ultracell ultrafiltration disc NMWL 10 kDa (Merck-Millipore, Darmstadt, Germany) and stored at -80 °C. Other FMN isotopologues ([2-$^{13}C_1$]-**10**, [4-$^{13}C_1$]-**10,** 10-$^{15}N_1$]-**10,** [3-$^{15}N_1$]-**10,** [4-$^{17}O_1$]-**10,** [4-$^{18}O_1$]-**10,** [5a,6,7,7α,8,8α,9,9a,1',2',3',4',5'-$^{13}C_{13}$]-**10**) were synthesized using the same protocol. The starting amounts of **7** were between 2 and 3 mg. The yield was between 95% and 97%.

[10a-$^{13}C_1$]-FMN from [8a-$^{13}C_1$]6,7-dimethyl-8-ribityllumazine. [8a-$^{13}C_1$]-**6** (10 mg) was suspended in 5 ml of 100 mM Tris hydrochloride, pH 7.6, containing 5 mM $MgCl_2$, 5 mM DTT, 0.02% NaN_3, 6.5 mM 3,4-dihydroxy-2-butanone 4-phosphate, 0.5 mM ATP, 7.5 mM sodium phosphoenol pyruvate, 10 units of lumazine synthase, 10 units of riboflavin synthase, 11 units of pyruvate kinase from rabbit muscle and 4 units of riboflavin kinase. The mixture was incubated at 37 °C for 12 h. The pH value of the reaction mixture was kept at 8.0 by the addition of 5 M NaOH as required. The progress of the reaction was monitored by thin layer chromatography (microcrystalline cellulose plates, Macherey-Nagel; eluent, 350 mM phosphate, pH 7.0). Typically, **6** was completely converted to FMN after 12 h. The solution was passed through an Ultracell ultrafiltration disc NMWL 10 kDa (Merck-Millipore, Darmstadt, Germany) and stored at -80 °C. Yield, 13 mg, 92 % based on proffered **6**.

[10a-$^{13}C_1$]-FAD. [10a-$^{13}C_1$]Riboflavin (44 mg) were resuspended in 18 ml of 100 mM Tris hydrochloride, pH 8.0, containing 5 mM $MgCl_2$, 5 mM DTT, 0.02% NaN_3, 8.5 mM ATP and 9 mM sodium phosphoenol pyruvate. Pyruvate kinase from rabbit muscle (15 units), 20 units of inorganic pyrophosphatase from baker's yeast and 12 units of bifunctional flavokinase/FAD synthetase were added. The suspension was incubated at 37 °C 12 h. The pH value of the reaction buffer was kept at 8.0 by addition of 5 M NaOH as required. The progress of the reaction was monitored visually (disappearance of riboflavin crystals) as well

as by thin layer chromatography (microcrystalline cellulose plates, Macherey-Nagel; eluent, 350 mM phosphate, pH 7.0). Typically, riboflavin was completely converted to FAD within 12 h. Yield, 86 mg, 95 % based on proffered riboflavin (mol per mol). The solution was passed through an Ultracell ultrafiltration disc NMWL 10 kDa (Merck-Millipore, Darmstadt, Germany) and stored at -80 °C. Other FAD isotopologues ([2-$^{13}C_1$]-**11**, [4-$^{13}C_1$]-**11,** 10-$^{15}N_1$]-**11,** [3-$^{15}N_1$]-**11,** [4-$^{17}O_1$]-**11,** [4-$^{18}O_1$]-**11,** [5a,6,7,7α,8,8α,9,9a,1',2',3',4',5'-$^{13}C_{13}$]-**11**) were synthesized using the same protocol. The starting amounts of **7** were between 2 and 3 mg. The yield was between 95% and 97%.

Alternatively, FAD could be prepared directly from **6** as follows. [8a-$^{13}C_1$]-**6** (10 mg) was suspended in 5 ml of 100 mM Tris hydrochloride, pH 7.6, containing 5 mM $MgCl_2$, 5 mM DTT, 0.02% NaN_3, 6.5 mM 3,4-dihydroxy-2-butanone 4-phosphate, 6.5 mM ATP and 7.5 mM sodium phosphoenol pyruvate. Lumazine synthase (10 units), 10 units of riboflavin synthase, 11 units of pyruvate kinase from rabbit muscle and 10 units of flavokinase/FAD synthetase were added and the mixture was incubated at 37 °C for 12 h. The pH value of the reaction mixture was kept at 8.0 by addition of 5 M NaOH as required. The progress of the reaction was monitored by thin layer chromatography (microcrystalline cellulose plates, Macherey-Nagel; eluent, 350 mM phosphate, pH 7.0). Typically, **6** was completely converted to FAD after 12 h. The solution was passed through an Ultracell ultrafiltration disc NMWL 10 kDa (Merck-Millipore, Darmstadt, Germany) and stored at -80 °C. Yield, 21.5 mg, 90 % based on proffered **6** (mol per mol).

Purification of FMN and FAD. The purification procedure is based on a method described earlier.[39] Aliquots (200 µL) containing 6 mg of the FMN or FAD were applied to a reverse phase column (Nucleosil C18, Macherey-Nagel, Düren, Germany, 250 × 21 mm) that had been equilibrated with 35% aqueous methanol containing 0.1 M ammonium formate. The column was developed with the same eluent at a flow rate of 15 ml/min (22 °C). The retention

times for FMN and FAD were 6.4 min and 5.3 min, respectively. Fractions were combined and methanol was removed by evaporation. The residue was lyophilized and stored at -20 °C.

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Supporting Information. LC-MS and NMR data of labeled riboflavin and 6,7-dimethyl-8-ribityllumazine samples (including signal assignments and numerical values for chemical shifts and coupling constants) are available free of charge via the Internet at http://pubs.acs.org

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